RESEARCH ARTICLE

SABRAO Journal of Breeding and Genetics
57 (6) 2648-2658, 2025
http://doi.org/10.54910/sabrao2025.57.6.37
http://sabraojournal.org/
pISSN 1029-7073; eISSN 2224-8978

GENOTYPIC VARIATION IN TERMS OF BIOMASS YIELD AND MICRONUTRIENTS' ACCUMULATION IN RESPONSE TO ZINC AND IRON APPLICATION IN ALFALFA

J. ZEGARRA[1*], A. OBANDO[2], F. CORNEJO[1], A. CONDORI[1], F. ORE[3*], R. BLAS[4], J. ALEGRE[5], and S. GARCÍA[5]

[1]Professional School of Agronomic and Agricultural Engineering, Catholic University of Santa María, Arequipa, Peru
[2]Professional School of Veterinary Medicine and Zootechnics, Catholic University of Santa María, Arequipa, Peru
[3]Academic Department of Agroindustrial Engineering, National University of Huancavelica, Huancavelica, Peru
[4]Department of Plant Breeding, National Agrarian University La Molina, Lima, Peru
[5]Department of Soils, National Agrarian University La Molina, Lima, Peru
Corresponding authors' emails: jzegarraf@ucsm.edu.pe, franklin.ore@unh.edu.pe
Email addresses of co-authors: aobandos@ucsm.edu.pe, 71844621@ucsm.edu.pe, 73948578@ucsm.edu.pe, rblas@lamolina.edu.pe, jalegre@lamolina.edu.pe, sjgarciab@lamolina.edu.pe

SUMMARY

With enhanced sustainability in food and feed production systems, agronomic biofortification is a key strategy to alleviate micronutrient deficiencies in animal diets, with indirect benefits for human nutrition through improved quality of animal-derived foods. A field experiment, carried out using a randomized complete block design with a split-plot arrangement, included two levels each of zinc (Zn, 0 and 2 kg ha^{-1}) and iron (Fe, 0 and 2 kg ha^{-1}) and their integrated application (2 kg Zn ha^{-1} + 2 kg Fe ha^{-1}) in alfalfa (*Medicago sativa* L.). The Zn and Fe foliar application enhanced the concentration of both micronutrients in alfalfa without compromising its yield and quality. Alfalfa cultivars responded differently to biofortification treatments, and the cultivar California 55 showed the highest recovery efficiency (RE) of Zn (119.4%) and Fe (68.0%) at the fifth harvest. An RE value above 100% indicates the applied nutrient both contributed directly to uptake and enhanced the mobilization or utilization of native soil reserves, leading to greater total accumulation than the amount applied. The results highlighted that foliar biofortification is an effective and sustainable approach to improve the nutritional quality of forage crops, thereby contributing to livestock health, food security, and agricultural resilience.

Keywords: Alfalfa (*M. sativa* L.), cultivars, agronomic biofortification, foliar fertilization, zinc, iron, micronutrients efficiency, yield and quality

Communicating Editor: Dr. Hida Arliani Nur Anisa

Manuscript received: June 21, 2025; Accepted: September 06, 2025.

Citation: Zegarra J, Obando A, Cornejo F, Condori A, Ore F, Blas R, Alegre J, García S (2025). Genotypic variation in terms of biomass yield and micronutrients' accumulation in response to zinc and iron application in alfalfa. *SABRAO J. Breed. Genet.* 57(6): 2648-2658. http://doi.org/10.54910/sabrao2025.57.6.37.

Key findings: Foliar applications of Zn and Fe significantly increased their concentration in alfalfa (*M. sativa* L.) without affecting its yield and quality. Zn and Fe foliar application enhanced the concentration of both micronutrients in alfalfa.

INTRODUCTION

Forage crops provide the primary source of minerals in livestock nutrition. However, soil and crop plants with natural deficiencies in micronutrients, such as zinc (Zn) and iron (Fe), can affect animal health and productivity. Among the various available strategies to address these issues, agronomic biofortification has emerged as a promising and sustainable approach to enrich the different forage crops with essential micronutrients, thereby improving feed quality and combating malnutrition in animals (Novoselec *et al.,* 2018). In turn, healthier livestock and higher-quality animal products can contribute indirectly to alleviating micronutrient deficiencies in human populations that rely on dairy and meat as dietary sources of Zn and Fe. A large portion of the global population suffers from health conditions associated with malnutrition due to zinc deficiency, including malabsorption syndrome, liver disease, chronic kidney disease, and sickle cell anemia (Gadapati, 2025). These issues mostly have a linkage to cereal-based diets, which are inherently low in Zn due to poor soil availability (Koren and Tako, 2020). Addressing these problems requires both dietary diversification and the biofortification of crops, which have become crucial strategies. In the case of alfalfa, despite its reputation as the "queen of forages" and its naturally high-protein content, micronutrient levels, such as Zn and Fe, can be suboptimal, particularly when grown in calcareous soils where deficiencies are widespread. This can limit the nutritional value of alfalfa for livestock, reducing animal growth, productivity, and ultimately, the micronutrient density of animal-derived foods consumed by humans. Thus, improving the Zn and Fe status of alfalfa through biofortification directly supports both livestock health and human nutrition.

Similarly, Zn deficiency alleviation in animals can succeed through Zn-enriched feed and supplements, ultimately improving livestock health and indirectly benefiting human health through higher-quality animal products (Shukla *et al.,* 2023). In crop plants, foliar application of Zn has generally appeared more effective than soil amendment. This is because Zn uptake by plant roots often has restrictions from low solubility of Zn salts, considerable binding to organic matter, and immobilization within the microbial biomass. In addition, iron is also a vital micronutrient required for numerous biological functions. Iron deficiency is most common in calcareous soils, comprising approximately one-third of the world's arable lands. Iron plays a pivotal role in photosynthesis and contributes to essential physiological processes such as chlorophyll synthesis, redox reactions, respiration, nitrogen and carbohydrate metabolism, and the maintenance of protoplasmic properties. Yet, like Zn, soil application of Fe is often ineffective. Ferrous iron (Fe^{2+}), when applied to soil, quickly oxidizes to ferric iron (Fe^{3+}), which is largely unavailable to plants.

Numerous studies have highlighted the effectiveness of foliar fertilization in biofortifying forage crops with micronutrients such as Zn and selenium (Se) (Petković *et al.,* 2019; Szerement *et al.,* 2022). For instance, foliar application of Zn (80 kg ha^{-1}) resulted in increased forage yield (30.9%), hay yield (34.7%), and dry matter yield (32.1%) compared with the control treatment. In pearl millet, grain yield improved by 31.5% with foliar Zn application, and Zn accumulation in grains reached 26.1 mg kg^{-1} with the combined application of 20 kg N and 5 kg Zn ha^{-1} (Prasad *et al.,* 2014; Singh *et al.*, 2019). Further, simultaneous foliar application of Zn, iodine (I), and Se effectively enhanced their concentrations in grains across multiple wheat cultivars grown under varied environmental conditions (*Zou et al.,* 2019). Various advanced and conventional techniques have been operational to improve Fe content in crops, including genetic engineering, traditional breeding, and agronomic biofortification. However, foliar fertilizers are practical, cost-effective, and highly efficient in promoting plant growth, particularly in soil deficient in these important nutrients. Foliar Fe fertilization has also been visible to positively affect the yield and quality in fruits, vegetables, and other crops.

Alfalfa (*Medicago sativa L.*), known as the queen of forages, is a valuable crop for livestock systems. The term 'alfalfa' originates from the Arabic word 'Al-Fasfasa,' meaning 'father of all plants.' Its reputation developed due to its high adaptability, considerable productivity potential, and superior forage quality. Alfalfa is particularly rich in protein (29%) and naturally contains substantial amounts of essential micronutrients, such as Zn (21 mg kg^{-1}) and Fe (30 to 250 mg kg^{-1}), and both these elements play a vital role in animal growth and development (Hermanto *et al.,* 2017). Limited research exists on the effects of Zn and Fe foliar fertilization in forage crops such as alfalfa. This research considered Zn and Fe's critical role in animal health and their demonstrated effectiveness as foliar fertilizers enhancing micronutrient levels in plants. Hence, this study aimed to determine the impact of foliar application of zinc and iron on yield, quality, and micronutrient levels of alfalfa biomass.

Although previous studies have explored Zn and Fe biofortification in cereal grains and legumes, limited research is available on their combined foliar application in forage crops such as alfalfa. Moreover, the role of genotypic variation in micronutrient uptake efficiency has not gained adequate attention. Therefore, the following study aimed to evaluate how foliar Zn and Fe application influences biomass yield, micronutrient accumulation, and recovery efficiency across different alfalfa cultivars.

MATERIALS AND METHODS

Study area and environmental conditions

The experiment transpired on a Typic Ustifluvents soil (Soil Survey Staff, 2022) at the Huasacache Experimental Station of the Universidad Católica de Santa María. The site is in the District of Jacobo Hunter, Arequipa Province, Peru (16°27′28.42″ S, 71°33′59.13″ W) at an elevation of 2209 m above sea level.

The arable soil layer (0–25 cm) had a loam texture with the following composition: sand (51%), silt (34%), and clay (15%). Other soil characteristics included pH - H_2O (8.10), calcium carbonate - $CaCO_3$ (4.71%), electrical conductivity (7.17 dS m^{-1}), organic matter (28.3 g kg^{-1}), available phosphorus - Olsen method (25.2 mg kg^{-1}), available potassium - extracted with NH_4OAc (421.8 mg kg^{-1}), cation exchange capacity - CEC (14.44 $cmol_{(C)}$ kg^{-1}), base saturation (100%), and extractable Zn and Fe using $NaHCO_3$ (2.29 and 9.16 mg kg^{-1}, respectively). During the study, the climatic conditions displayed maximum temperature ranges (24.2 °C to 21.7 °C) between September and April and minimum monthly temperatures (11.6 °C to 6.6 °C) between May and August. Relative humidity varied from 69% to 45% during the same periods. Precipitation showed more concentrations between January and February, with a daily maximum rainfall reaching up to 22 mm and a minimum around 12 mm (SENAMHI, n.d.) (Figure 1).

Plant material and experimental conditions

The sowing of four alfalfa (*M. sativa* L.) cultivars, Cuf 101, Moapa 69, California 55, and Yaragua, occurred on March 26, 2022, at a seeding rate of 25 kg ha^{-1} (based on seed weight), aiming to achieve a plant density between 40 and 70 plants per square meter. Each cultivar's sowing in plots had the measurement of 14.34 m × 5.5 m (78.87 m^2) and a layout in a randomized complete block design with split-plot arrangement, comprising five blocks, with each block containing all four cultivars.

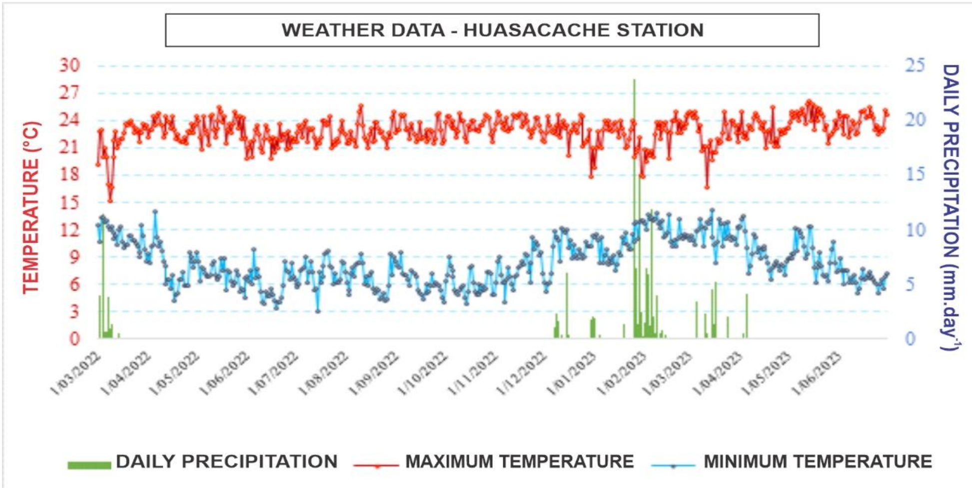

Figure 1. Temperature and relative humidity data obtained from the Huasacache Meteorological Station, Arequipa, Peru. (Source: SENAMHI n.d.)

Fertilization and experimental treatments

A basal fertilization dose of 20-25-50 kg ha^{-1} of N, P_2O_5, and K_2O, respectively, succeeded in applying before sowing. The fertilizers used were ammonium nitrate (35.5% N), diammonium phosphate (18% N and 46% P_2O_5), and potassium chloride (60% K_2O). Irrigation proceeded weekly by surface gravity using canal water.

The Zn and Fe application via foliar spraying to each alfalfa cultivar had the following rates: D1 (control, no application), D2 (Zn at 2 kg ha^{-1}), D3 (Fe at 2 kg ha^{-1}), and D4 (Zn 2 kg ha^{-1} + Fe 2 kg ha^{-1}). The application rate of 2 kg ha^{-1} was the selected basis because soil analysis indicated low baseline availability of Zn (2.29 mg kg^{-1}) and Fe (9.16 mg kg^{-1}). Likewise, previous studies have shown that foliar applications in this range are effective in correcting micronutrient deficiencies without causing toxicity (Fageria et al., 2009; Stewart et al., 2021).

The experiment, laid out in a randomized complete block design, comprised a split-plot arrangement and two factors, i.e., main plots: alfalfa cultivars (Cuf 101, Moapa 69, California 55, and Yaragua) and subplots: foliar treatments (control, Zn, Fe, and Zn + Fe). Each treatment consisted of five replicates after every harvest, resulting in experimental units totaling 80.

Evaluation of biometric variables

Plant height measurement ensued 40 days after each harvest (six harvests in total, including the first 'clean-up' harvest) on 10 randomly selected plants per plot (19.71 m²). Plant height underwent appraisal from the base of the crown to the tip of the longest extended leaf using a measuring tape. The counting of the number of stems per crown commenced on the same dates and in the same sampled plants. Biomass yield evaluation within each subplot used a 0.56 m² quadrat. Measurements proceeded starting 150 days after emergence (first harvest) and then, after every 40 days over a five-month period, totaling six harvests. The forage harvesting left a 5-cm regrowth height, with the fresh weight recorded using an analytical scale. Fresh biomass yield's expression was in kg/m². Approximately 300 g of fresh foliage, taken from each plot, succeeded in washing in running water and then in acidulated and deionized water before being oven-dried at 70 °C to constant weight and determining the dry matter content.

These biometric variables (height, stems, and leaves) were the basis for selection because they are key morphological traits directly linked to forage yield and quality. Plant height reflects overall vegetative growth and biomass accumulation, while stem number has an association with stand density and regrowth potential. Leaf number is particularly important for forage quality, since leaves contain higher protein and micronutrient concentrations than stems, contributing significantly to feed value for livestock (Capstaff and Miller, 2018).

Biochemical analysis of the alfalfa crop

a. Zinc and iron content in plant tissues

Tissue analysis, as carried out, used the protocols established by the Agricultural Chemistry Laboratory at the Institute of Rural Valle Grande, Cañete, Peru. One gram of oven-dried foliage from each plot underwent fine grinding with an electric mill before digestion in a 5:1 mixture of nitric and perchloric acid, following the method of Zasoski and Burau (1977). Digests received dilution to 50 mL with distilled water, with Zn and Fe concentrations determined using atomic absorption spectrophotometry.

b. Zinc and iron uptake in aerial biomass

In aerial biomass, the Zn and Fe uptake calculation continued by multiplying the dry biomass yield of the harvested shoots by the corresponding Zn and Fe concentrations determined in the tissue analysis.

c. Apparent nutrient recovery efficiency

The apparent recovery efficiency (RE) of Zn and Fe entailed calculation using the formula as described by González *et al*. (2008).

$$RE = (\text{Zn and Fe uptake in TX} - \text{Zn and Fe uptake in T0}) \div (\text{Amount of nutrient applied}) \times 100$$

Where TX represents the treatment in which 2 kg ha^{-1} of either Zn or Fe were applied and T0 represents the control treatment with 0 kg ha^{-1} of Zn and Fe. Both the uptake and the amount of applied nutrient's expression were in kg ha^{-1}. The uptake of Zn and Fe's calculation was the sum of the Zn and Fe accumulated in the aerial biomass across all the harvests of alfalfa. Note: RE values greater than 100% are possible because applied micronutrients may stimulate plant root activity, improve nutrient translocation, or enhance the mobilization of native soil reserves, leading to total uptake exceeding the amount directly applied.

Statistical analysis

The data recorded based on field variables and micronutrient concentrations sustained analysis of variance (ANOVA). Performing means comparison and separation used Tukey's honestly significant difference (HSD) test. The statistical analyses engaged the RStudio interface and the Agricolae package in the R statistical computing environment, version 4.2.3 (R Core Team, 2023). The corresponding graphs, assumption checks, and correlation analysis were successful using the ExpDes, Corrplot, ggplot2, and supporting libraries. No data transformations were necessary, as all datasets met these assumptions. Before the ANOVA, statistical assumptions entailed verification, including normality of residuals (Shapiro-Wilk test), homogeneity of variance (Levene's test), and independence of errors based on residual plots.

RESULTS

The results for plant height and dry matter yield for four alfalfa cultivars at the fifth harvest appear in Table 1. Cultivar Yaragua showed the highest values for plant height and dry matter yield (80.0 cm and 4877.9 kg/ha, respectively). Compared with the average of the other three cultivars (plant height = 65.9±0.89 cm and dry matter yield = 4,366.7±266.8 kg/ha), cultivar Yaragua's plant height was significantly greater (t = 27.4, P < 0.001) with a massive effect size (Cohen's d = 15.84). Additionally, its dry matter yield was

Table 1. Alfalfa cultivars' mean performance for agronomic traits across five harvests.

Cultivars	Plant height (cm)	Dry matter yield (kg/ha)	t-value (Yaragua vs. Others)	Cohen's d (Yaragua vs. Others)	Significance and effect size
Cuf 101	66.2	4123.3			
Moapa 69	64.9	4325.1			
California 55	66.6	4651.6			
Yaragua	80.0	4877.9			
Others (mean ± SD)	65.9±0.89	4,366.7±266.8			
Comparison (Yaragua vs. Others)	—	—	Plant height: 27.4	14.34	P < 0.001, Massive effect
			Dry matter yield: 3.32	1.92	P < 0.05, Large effect

significantly higher (t = 3.32, P < 0.05) with a large effect size (Cohen's d = 1.92). In practical terms, these effect sizes indicate that Yaragua's advantage in height and yield was statistically significant as well as of substantial agronomic importance, reflecting meaningful differences that would be visible and impactful under field conditions. The results revealed that alfalfa cultivar Yaragua outperformed the other three cultivars in growth and biomass production at this stage.

Plant height and stem count

The four alfalfa cultivars revealed significant differences in plant height. The cultivar Yaragua exhibited the tallest plant height, measuring 64.9, 69.2, 63.5, and 80.0 cm for the second, third, fourth, and fifth harvests, respectively (Table 1). A non-significant effect of foliar Zn and Fe applications on plant height was evident across all the alfalfa cultivars and harvests (Table 1). The results disclosed that alfalfa cultivar Yaragua outperformed the other three cultivars in growth and biomass production at this stage. These results confirm that Yaragua possesses a superior biomass productivity potential.

Stem numbers also differed significantly among the alfalfa cultivars during the first, third, fourth, and fifth harvests (Figure 2). Overall, stem count increased with plant age and successive harvests. Foliar treatments of Zn and Fe notably affected and enhanced the stem number at the first and fifth harvests. The cultivar Cuf 101 showed the highest stem count during the second and third harvests (mean ~5 stems), whereas cultivar Moapa 69 had considerably higher stem numbers in the fourth and fifth harvests (with means of 10 and 13 stems, respectively).

Number of leaves

For number of leaves, remarkable differences appeared among the alfalfa cultivars across all five harvests (Figure 3). Cultivar Moapa 69 exhibited the most number of leaves, with values of 85, 204, 196, 338, and 390 leaves from the first to the fifth harvest, respectively, surpassing the other cultivars. The number of leaves also increased progressively with each subsequent harvest. Foliar applications of Zn and Fe showed significant effects on leaf number during the first and fifth harvests. Specifically, the foliar application of 2 kg Fe ha^{-1} resulted in 362 leaves at the fifth harvest, which was considerably higher than other treatments.

Zinc and iron concentrations

The foliar zinc and iron concentrations varied significantly among the alfalfa cultivars at the fifth harvest (Table 2). Cultivar California 55 accumulated the maximum zinc concentration (240.4±18.2 mg/kg), which was essentially greater than that in the cultivars Cuf 101 and Yaragua, and both cultivars had lower zinc levels (around 150 mg/kg). Cultivar Moapa 69 emerged with intermediate zinc content. Regarding iron concentration, cultivar Moapa 69 displayed the highest accumulation (284.0±12.6 mg/kg), which substantially

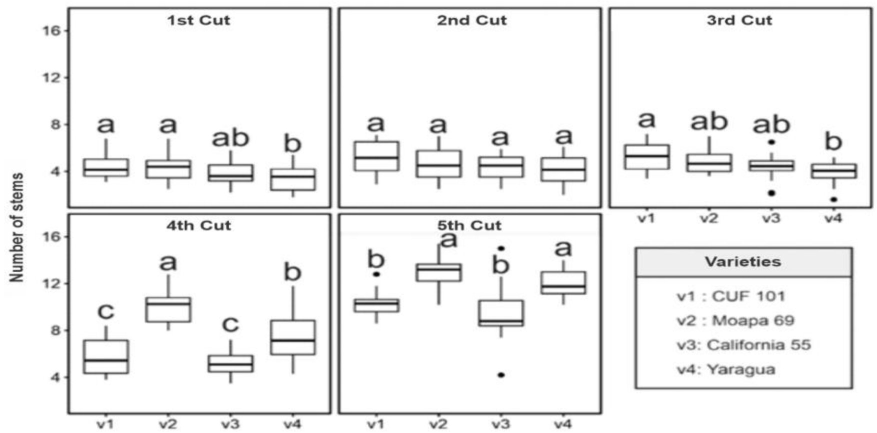

Figure 2. Effect of foliar application of four treatments (Control, Zn, Fe, and Zn + Fe) on the stem number in four alfalfa cultivars (Cuf 101, Moapa 69, California 55, and Yaragua) across five harvests. Data represent the mean values per treatment and the cultivar across successive harvests. Error bars indicate the standard error.

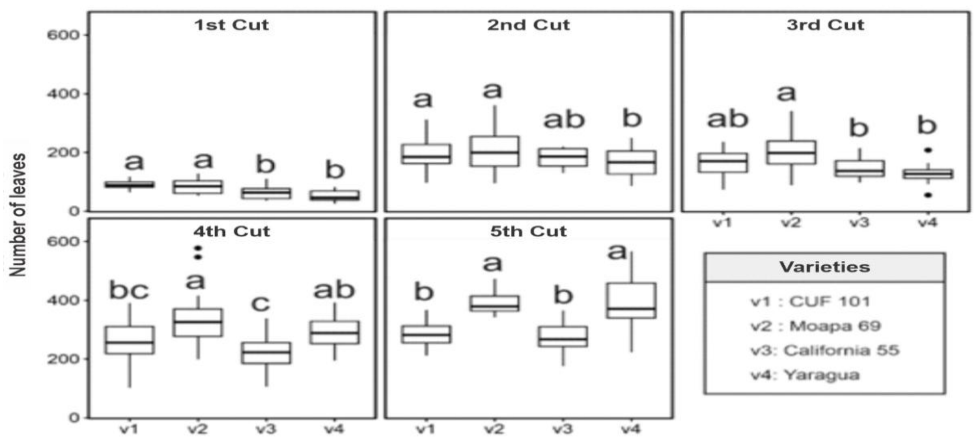

Figure 3. Effect of foliar application of four treatments (Control, Zn, Fe, and Zn + Fe) on the number of leaves in four alfalfa cultivars (Cuf 101, Moapa 69, California 55, and Yaragua) across five harvests. Values represent the mean leaf counts per treatment and the cultivar over successive harvests. Error bars indicate the standard error.

surpassed the cultivars Cuf 101 and Yaragua, recorded with the same and lower iron levels. In the cultivar California 55, the iron content was comparable to the cultivars Cuf 101 and Yaragua; however, it was noticeably lower than the cultivar Moapa 69. The results indicated obvious genotypic differences for micronutrient accumulation under the applied treatments.

The foliar treatments significantly influenced the zinc and iron concentrations in alfalfa cultivars at the fifth harvest (Table 3). The Zn-only treatment (D2) resulted in the highest zinc concentration (385.1±21.3 mg/kg) and occurred considerably more than all other treatments, including the combined application of Zn + Fe (D4). The control (D1) and Fe-only treatment (D3) showed the minimum levels of zinc concentration. Conversely, for iron concentration, the Fe-only treatment (D3) and combined application of Zn

Table 2. Foliar zinc and iron concentrations at the fifth harvest in alfalfa cultivars and treatments.

Cultivars	Zn (mg/kg) Mean ± SD	Fe (mg/kg) Mean ± SD
Cuf 101	150.5±12.3a	210.1±10.7b
Moapa 69	196.0±15.1b	284.0±12.6c
California 55	240.4±18.2c	268.0±11.9bc
Yaragua	146.9±13.0a	212.8±9.8b

Table 3: Zinc and iron concentrations with different treatments.

Treatments	Zn (mg/kg) Mean ± SD	Fe (mg/kg) Mean ± SD
D1 (Control)	36.6±4.2a	147.6±8.50a
D2 (Zn: 2 kg/ha)	385.1±21.3c	138.1±7.20a
D3 (Fe: 2 kg/ha)	42.7±3.7a	345.7±19.0d
D4 (Zn + Fe: 2+2)	269±17.5b	343.6±16.8d

Note: Different letters within columns indicate statistically significant differences (Tukey's HSD, $P < 0.05$).

Table 4. Apparent recovery efficiency (RE %) of zinc and iron at the fifth harvest in alfalfa cultivars and treatments.

Cultivars	Zn RE (%) (D2) Mean ± SD	Fe RE (%) (D4) Mean ± SD
Cuf 101	49.3±3.5b	33.1±2.7b
Moapa 69	88.6±5.1c	54.2±4.0c
California 55	119.4±7.0d	68.0±5.5d
Yaragua	68.4±4.8bc	26.9±2.2a

+ Fe (D4) produced the maximum iron contents (345.7±19.0 and 343.6±16.8 mg/kg, respectively). Both treatments significantly surpassed the control and Zn-only treatment. These results highlighted the effectiveness of targeted foliar fertilization in enhancing specific micronutrient accumulation in alfalfa plant leaves.

The findings enunciated that the apparent recovery efficiency (RE) of zinc and iron varied notably among the alfalfa cultivars under specific foliar treatments (Table 4). Cultivar California 55 demonstrated the highest zinc recovery efficiency (119.4% ± 7.0%), significantly outperforming all other cultivars, followed by cultivar Moapa 69 with a substantial zinc RE (88.6% ± 5.1%). However, the alfalfa cultivars Yaragua and Cuf 101 showed the moderate zinc RE values. For iron recovery efficiency, cultivar California 55 again led with the topmost value (68.0% ± 5.5%), and cultivar Moapa 69 also showed a strong recovery (54.2% ± 4.0%). Cultivars Yaragua and Cuf 101 expressed the lowest iron RE, with cultivar Yaragua having the lowest value. In contrast, Yaragua and Cuf 101 gave lower efficiencies. These findings confirm substantial genotypic variation in nutrient uptake efficiency, consistent with earlier reports on crop species where recovering foliar-applied micronutrients was more efficient with specific genotypes. These results reflected considerable genotypic variations in nutrient uptake efficiency, especially highlighting that alfalfa cultivar California 55 was the most efficient in recovering foliar-applied zinc and iron.

DISCUSSION

Alfalfa cultivar Yaragua was visible with taller plant height, reflecting its genetic potential and cultivar-specific trait. However, no further enhancement in plant height from foliar application of Zn and Fe suggested that soil nutrient levels and their uptake mechanisms were sufficient for growth during the study. Zinc is essential for nucleic acid stability and hormone biosynthesis (Cakmak, 2000), as well as enzymatic activities associated with photosynthesis and cell division. Stem number varied among the alfalfa cultivars and gradually increased with harvests, consistent with known alfalfa development patterns (JiShan *et al.,* 2013). Foliar application of Zn and Fe appeared to stimulate stem production, possibly by enhancing hormonal activity. Alfalfa enriched with essential micronutrients contributes to improved mineral nutrition in animals, which, in turn, enhances growth, reproduction, and the quality of animal-derived products such as milk and meat. At a larger scale, this strategy represents a sustainable and cost-effective approach to address micronutrient deficiencies in livestock production, reducing the need for external mineral supplementation and supporting food security goals (Cakmak, 2000).

Although the results demonstrate clear benefits of foliar Zn and Fe application, the study proceeded under specific environmental and soil conditions. Micronutrient uptake efficiency may vary under different soil types, rainfall regimes, or management practices (Cakmak, 2008). Furthermore, foliar application efficiency could bear influences from climatic factors, such as humidity, temperature, and rainfall patterns during application, which may alter nutrient absorption through the leaf surface (Julier *et al.,* 2000). Another aspect to consider is the sustainability of the observed improvements across multiple growing seasons. Even though this study covered successive harvests within one season, it remains unclear whether repeated foliar applications over several years would maintain or further enhance nutrient accumulation and biomass yield. Moreover, it is unsure whether diminishing returns would occur due to nutrient interactions or soil feedback mechanisms.

High-yielding alfalfa cultivars like Cuf 101 and Moapa 69 showed favorable traits for biomass production across environmental conditions (Julier *et al.,* 2000). The yield increases in the second and subsequent years were apparent as root systems matured (Wang *et al.,* 2009). Cultivar Cuf 101 outperformed others in dry matter yield, while cultivar California 55 exceeded cultivar Moapa 69 (Marble, 1997). Zn application has been shown to improve alfalfa yields, although higher yields often dilute Zn concentration in the plant tissues (Davis, 2011). These presented results were consistent with reports that foliar Zn, Fe, and Mn improve the forage yield and quality (Ghanbari *et al.,* 2010). The superior recovery efficiency observed in cultivar California 55 may refer to genotypic traits that enhance nutrient uptake and translocation. For Zn, efficient genotypes typically show association with greater root surface area, higher exudation of organic acids, and increased expression of Zn transporters that improve solubility and uptake (Cakmak, 2008; Kawakami and Bhullar, 2018). Similarly, efficient Fe uptake has links to the production of root exudates such as phytosiderophores and enhanced ferric-chelate reductase activity, which facilitate Fe mobilization under alkaline soil conditions. Once absorbed, efficient cultivars may also allocate more micronutrients into leaves, where they participate in metabolic functions such as chlorophyll synthesis and enzyme activation (Dhaliwal *et al.,* 2022). The performance of California 55 in this study suggests that it may combine effective foliar absorption with superior internal utilization efficiency, making it a promising candidate for biofortification-oriented breeding programs (Dhaliwal *et al.,* 2022).

Critical Zn leaf concentration varies between 100 and 700 mg/kg DM (Fageria *et al.,* 2009); however, foliar application can achieve higher Zn without yield loss (Cakmak, 2008). Variation in Zn accumulation among the alfalfa cultivars was significant, consistent with wide genotypic variability, with the same also reported in other crops (Grusak and Cakmak, 2005). This suggests breeding for improved Zn content is feasible and that genotypes differed in Zn utilization efficiency. Foliar Zn fertilization increased leaf Zn concentration across the harvests, showing a considerable correlation with prior studies (Capstaff and Miller, 2018).

The Zn deficiency threshold (~15–20 µg/g DM) was exceeded in all the alfalfa cultivars after application (Alloway, 2012). Foliar Zn and Fe applications significantly boost the nutrient content by direct leaf uptake, consistent with past findings in wheat and rice (Dhaliwal *et al.,* 2021). Combined Zn and Fe application suggested shared translocation pathways (Kawakami and Bhullar, 2018), and the nutrient uptake had no inhibition from co-application (Stewart *et al.,* 2021). Overall, foliar application of the micronutrients proved effective in enhancing Zn and Fe content in alfalfa foliage.

Future research

Future research should also explore whether repeated foliar Zn and Fe applications across several years lead to sustained improvements in yield and nutritional content or if diminishing returns and nutrient interactions occur over time. Such studies would provide critical insights for scaling up biofortification strategies in diverse production environments.

CONCLUSIONS

Alfalfa cultivar Yaragua demonstrated increased plant height due to its genetic potential, while foliar application of Zn and Fe nutrients had a nonsignificant effect on its plant height; however, it enhanced its stem production and nutrient content. Foliar fertilization effectively increased Zn and Fe concentrations in alfalfa leaves, surpassing deficiency thresholds without inhibiting uptake when applied together. Significant genetic variations in micronutrient accumulation suggested the potential for breeding programs targeting nutrient efficiency. Overall, foliar applications of Zn and Fe improved forage yield and quality and micronutrient contents, supporting their use in alfalfa cultivation for better nutritional outcomes.

REFERENCES

Alloway BJ (2012). Heavy Metals in Soils: Trace Metals and Metalloids in Soils and their Bioavailability. Springer Science and Business Media.

Cakmak I (2000). Tansley Review No. 111 Possible roles of zinc in protecting plant cells from damage by reactive oxygen species. *New Phytol.* 146(2): 185-205.

Cakmak I (2008). Enrichment of cereal grains with zinc: Agronomic or genetic biofortification? *Plant Soil* 302(1): 1-17. https://doi.org/10.1007/s11104-007-9466-3.

Capstaff NM, Miller AJ (2018). Improving the yield and nutritional quality of forage crops. *Front. Plant Sci.* 9. https://www.frontiersin.org/articles/10.3389/fpls.2018.00535.

Davis DR (2011). Impact of breeding and yield on fruit, vegetable, and grain nutrient content. In: Breeding for Fruit Quality, pp. 127-150.

Dhaliwal SS, Sharma V, Shukla AK, Kaur J, Verma V, Singh P, Singh H, Abdel-Hafez SH, Sayed S, Gaber A, Ali R, Hossain A (2021). Enrichment of zinc and iron micronutrients in lentil (*Lens culinaris* Medik.) through biofortification. *Molecules* 26(24). https://doi.org/10.3390/molecules26247671.

Dhaliwal SS, Sharma V, Shukla AK, Verma V, Kaur M, Shivay YS, Hossain A (2022). Biofortification – a frontier novel approach to enrich micronutrients in field crops to encounter the nutritional security. *Molecules* 27(4): 1340.

Fageria NK, Filho MPB, Moreira A, Guimarães CM (2009). Foliar fertilization of crop plants. *J. Plant Nutr.* 32(6): 1044-1064. https://doi.org/10.1080/01904160902872826.

Gadapati S (2025). Global health burden of zinc deficiency. In: Strategies to Alleviate Human Zinc Deficiency, IGI Global Scientific Publishing, pp. 65-90.

Ghanbari A, Dahmardeh M, Fakheri B, Ramroudi M (2010). Effect of maize (*Zea mays* L.)—cowpea (*Vigna unguiculata* L.) intercropping on light distribution, soil temperature, and soil moisture in arid environment. *J. Food Agric. Environ.* 8(1): 102-108.

Grusak MA, Cakmak I (2005). Methods to Improve the Crop-Delivery of Minerals to Humans and Livestock.

Hermanto H, Suwignyo B, Umami N (2017). Kualitas kimia dan kandungan klorofil tanaman alfalfa (*Medicago sativa* L.) dengan lama penyinaran dan dosis dolomit yang berbeda pada tanah regosol. *Bull. Peternakan* 41(1): 54-60.

JiShan C, Cao G, GuiLi D, RuiFen Z, YueXue Z (2013). Effects of cutting on alfalfa yield and quality in Northeast China. *J. Anim. Vet. Adv.* 12(2): 253-260. https://www.cabdirect.org/cabdirect/abstract/20133294145.

Julier B, Huyghe C, Ecalle C (2000). Within- and among-cultivar genetic variation in alfalfa: Forage quality, morphology, and yield. *Crop Sci.* 40(2): 365-369. https://doi.org/10.2135/cropsci2000.402365x.

Kawakami Y, Bhullar NK (2018). Molecular processes in iron and zinc homeostasis and their modulation for biofortification in rice. *J. Integr. Plant Biol.* 60(12): 1181-1198. https://doi.org/10.1111/jipb.12751.

Koren O, Tako E (2020). Chronic dietary zinc deficiency alters gut microbiota composition and function. *Proc.* 61(1). https://doi.org/10.3390/IECN2020-06993.

Marble VL (1977). Alfalfa variety characteristics and adaptation in California. In: Proceedings Seventh California Alfalfa Symposium, pp. 11-21.

Novoselec J, Klir Ž, Domaćinović M, Lončarić Z, Antunović Z (2018). Biofortification of feed stuffs with microelements in animal nutrition. *Poljoprivreda* 24(1): 25-34.

Petković K, Manojlović M, Lombnæs P, Lončarić Z (2019). Foliar application of selenium, zinc and copper in alfalfa (*Medicago sativa* L.) biofortification. *Turkish J. Field Crops* 24(1): 81-90.

Prasad SK, Singh R, Singh MK, Rakshit A (2014). Zinc biofortification and agronomic indices of pearl millet under semi-arid region. *Int. J. Agric. Environ. Biotechnol.* 8(1): 171. https://doi.org/10.5958/2230-732X.2015. 00022.4

SENAMHI (n.d.). Datos hidrometeorológicos a nivel nacional SENAMHI - Perú. Retrieved June 16, 2023 from https://www.senamhi.gob. pe/?p=estaciones.

Shukla M, Prajapati B, Pathak Y (2023). Biofortification: A new tool for healthier future of India. *Recent Adv. Agric. Sci. Technol.* 2: https://www.researchgate.net/ publication/371165330_Biofortification_A_Tool_for_ Improve_the_Food_Security_and_ Nutritional_Security_in_India.

Singh C, Singh B, Satpal PK, Ankush M, Gora K, Kumar A (2019). Micronutrient management for enhancing production of major fodder crops: A review. *Forage Res.* 45(2): 95-102.

Stewart ZP, Paparozzi ET, Wortmann CS, Jha PK, Shapiro CA (2021). Effect of foliar micronutrients (B, Mn, Fe, Zn) on maize grain yield, micronutrient recovery, uptake, and partitioning. *Plants* 10(3): 528. https://www.mdpi.com/2223-7747/10/3/528.

Szerement J, Szatanik-Kloc A, Mokrzycki J, Mierzwa-Hersztek M (2022). Agronomic biofortification with Se, Zn, and Fe: An effective strategy to enhance crop nutritional quality and stress defense: A review. *J. Soil Sci. Plant Nutr*. 22(1): 1129-1159.

Wang C, Ma BL, Yan X, Han J, Guo Y, Wang Y, Li P (2009). Yields of alfalfa varieties with different fall-dormancy levels in a temperate environment. *Agron. J.* 101(5): 1146-1152. https://doi.org/10.2134/agronj2009.0026

Zasoski RJ, Burau RG (1977). A rapid nitric-perchloric acid digestion method for multi-element tissue analysis. *Commun. Soil Sci. Plant Anal.* 8: 425-436.

Zou C, Du Y, Rashid A, Ram H, Savasli E, Pieterse PJ, Ortiz-Monasterio I, Yazici A, Kaur C, Mahmood K, Singh S, Le Roux MR, Kuang W, Onder O, Kalayci M, Cakmak I (2019). Simultaneous biofortification of wheat with zinc, iodine, selenium, and iron through foliar treatment of a micronutrient cocktail in six countries. *J. Agric. Food Chem.* 67(29): 8096-8106. https://doi.org/10.1021/acs.jafc.9b01829.